Infection, Genetics and Evolution 75 (2019) 103991

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Analysis of GII.P7 and GII.6 noroviruses circulating in Italy during 2011–2016 reveals a replacement of lineages and complex recombination history

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ARTICLE INFO

Keywords:
Norovirus
GII.6
GII.P7
Genotype
Capsid
Polymerase

ABSTRACT

Noroviruses are important human enteric pathogens and monitoring their genetic diversity is important for epidemiological surveillance, vaccine development, and understanding of RNA viruses evolution. Epidemiological investigations have revealed that genogroup II, genotype 6 noroviruses (GII.6) are common agents of gastroenteritis. Upon sequencing of the ORF2 (encoding the viral capsid), GII.6 viruses have been distinguished into three variants. Sentinel hospital-based surveillance in Italy revealed that GII.6 noroviruses were the second most common capsid genotype in 2015, mostly in association with a GII.P7 ORF1 (encoding the viral polymerase). Upon molecular characterization of the ORF1 and ORF2, the GII.P7_GII.6 epidemic viruses circulating in 2014–2015 (variant GII.6b) were different from those that circulated sporadically in 2011–2013 (variant GII.6a). Analysis of the ORF1 (GII.P7) and ORF2 (GII.6) sequences available in the databases unveiled marked genetic diversity and peculiarities in the phylogenetic segregation patterns, suggesting multiple recombination events. Phylogenetic analyses suggest that recent GII.P7_GII.6b viruses were circulating as early as 2008, and formed a genetically homogenous group that emerged globally.

1. Introduction

Noroviruses are a major cause of acute gastroenteritis (AGE) in children and adults worldwide (Ahmed et al., 2014). Noroviruses are small non-enveloped round viruses with a single-stranded RNA genome of about 7.5 kb in length. The ORF1 encodes for non-structural proteins, including the viral RNA-dependent RNA-polymerase, whilst ORF2 and ORF3 encode for the major capsid protein and a minor capsid protein, respectively (Green, 2013).

Based on the ORF2, noroviruses are classified into at least seven genogroups (GI to GVII) with genogroup GI, II and IV causing disease in humans. Human noroviruses within genogroups GI, GII and GIV can be further classified into > 30 genotypes. Also, a classification system for the ORF1 has been proposed for GI and GII human noroviruses (Kroneman et al., 2013; Vinjé, 2015). During the last 2–3 decades symptomatic infection in humans has been mostly linked to a single genotype, GII.4. Noroviruses evolve through accumulation of point mutations and recombination (Eden et al., 2013; Parra et al., 2017). Recombination generates novel norovirus strains and may occur throughout norovirus genome (Begall et al., 2018), although a common hot spot for recombination is the ORF1/ORF2 junction region (Bull et al., 2005).

More recently, novel non-GII.4 norovirus strains have been observed as predominant genotypes in children hospitalized due to acute gastroenteritis. During the 2014/2015 winter season a novel GII.P17_GII.17 norovirus strain, Kawasaki 2014, emerged in several

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https://doi.org/10.1016/j.meegid.2019.103991
Received 25 May 2019; Received in revised form 1 August 2019; Accepted 2 August 2019
Available online 05 August 2019

Table 1
Nucleotide sequences length of norovirus strains analysed in this study.

	ORF1[a]	ORF1/2	ORF2	ORF2/3	ORF3	3'UTR	Sequence length[b]	Polymerase	Capsid	Accession
528/15-BA11/14	826	20	1644	1	777	52	3278	GII.P7	GII.6b	MH279838
613-15-BA3/15	812	20	1644	1	777	52	3264	GII.P7	GII.6b	MH279835
613/15-BA8/15	826	20	1644	1	777	52	3278	GII.P7	GII.6b	MH279836
613/15-BA16/14	826	20	1644	1	777	52	3278	GII.P7	GII.6b	MH279837
886/15-BA7/15	826	20	1644	1	777	52	3278	GII.P7	GII.6b	MH279839
159/16-PR3020/15	826	20	1644	1	777	53	3279	GII.P7	GII.6b	MH279827
159/16 PR4741/15	807	20	1644	1	774	48	3252	GII.P7	GII.6b	MH279828
150/16-PA309/11	826	20	1653	1	780	53	3291	GII.P7	GII.6a	MH279832
150/16-PA455/12	797	20	1653	1	780	53	3262	GII.P7	GII.6a	MH279834
150/16-PA226/13	827	20	1653	1	780	51	3290	GII.P7	GII.6a	MH279831
150/16-PA154/07	827	20	1623	1	774	70	3273	GII.P7	GII.7	MH279833
150/16-PA133/08	826	20	1623	1	774	70	3272	GII.P7	GII.7	MH279830
150/16-PA91/12	826	20	1623	1	774	68	3270	GII.P7	GII.7	MH279829
202/16-12	805	20	1611	1	795	46	3236	GII.P7	GII.14	MH279826

[a] Partial.
[b] Without the poly-A tail.

Table 2
Sequence set defined by co-phylogenetic analysis of ORF1 and ORF2 of GII.6 viruses (seq 1 to 6) and representative Italian GII.6 viruses (seq 7 to 10) used for recombination and sequence classification analysis.

Sequence type	Polymerase/capsid	Strain	Accession
Seq1	GII.P7/GII.6b	US/2012/BethesdaD1	KY424341
Seq2	GII.P7/GII.6b	US/1997/E99-13646	GU930737
Seq3	GII.P6/GII.6b	JP/2002/Saitama U16	AB039778
Seq4	GII.P7/GII.6c	US/2010/HS245	KJ407072
Seq5	GII.P6/GII.6c	US/1971/HenrytonH1	KY424345
Seq6	GII.P7/GII.6a	US/1984/CHDC4073	JX846927
Seq7	GII.P7/GII.6a	IT/2011/150/16-PA309	MH279832
Seq8	GII.P7/GII.6a	IT/2012/150/16-PA455	MH279834
Seq9	GII.P7/GII.6b	IT/2015/150/16-PR4741	MH279828
Seq10	GII.P7/GII.6b	IT/2015/886/15-BA7	MH279839

Asian countries to replace the pandemic strain GII.4 Sydney 2012 (Chan et al., 2015; Lu et al., 2015; Matsushima et al., 2015; Zhang et al., 2015), and subsequently spread to countries outside Asia (de Graaf et al., 2015). Moreover, during the 2015/2016 winter season, recombinant norovirus strains with a GII.P16 ORF1 (polymerase) and a GII.2 ORF2 (capsid) type emerged globally (Niendorf et al., 2017; Thongprachum et al., 2017; Lun et al., 2018), whilst GII.P16_GII.4 viruses were predominant in US (Cannon et al., 2017). The epidemiological relevance of non-GII.4 noroviruses poses challenges for the development of vaccines, as it is not clear to what extent, the antigens (GI.1 and GII.4) included in the vaccines under development (Cortes-Penfield et al., 2017) would be able to protect against other noroviruses (Parra et al., 2017; Bányai et al., 2018).

Between 2014 and 2016, the Italian Study Group for Enteric Viruses (ISGEV; http://isgev.net) observed the sustained circulation of GII.6 norovirus strains associated with a GII.P7 polymerase type. In 2015, genotype GII.6 accounted for 10% of norovirus infections, whilst before 2014 it was identified only sporadically. Circulation of GII.6 viruses has also been reported elsewhere in recent years (Chan et al., 2015; Bruggink et al., 2017). During 2014–2015, 10.3% (94/910) of the reported norovirus outbreaks in US were caused by GII.6 (Cannon et al., 2017). Between 2014 and 2016, GII.6 noroviruses accounted for 2–7.1% of the norovirus cases reported yearly by Noronet in European and collaborating countries outside Europe (van Beek et al., 2018). A review of norovirus data between 2004 and 2012 from 37 studies revealed that, overall, GII.6 viruses were the third most common capsid type (3.6%) after GII.3 (16.3%) and GII.4 (67.2%). Some studies showed that GII.6 viruses were the second most common capsid type, likely due to spatio-temporal variations (Hoa Tran et al., 2013). These findings suggest that this genotype has an important epidemiological role in norovirus incidence, and the molecular/biological mechanisms of its prevalence have not been addressed in detail. Upon sequence and phylogenetic analysis based on the whole ORF2 sequence, GII.6 viruses have been shown to cluster in three large groups, namely GII.6a, GII.6b and GII.6c (Chan-It et al., 2012; Vinjé, 2015). Strains within each of these groups or variants tend to be conserved as they display < 5% amino acid intra-group variation (Parra et al., 2017).

In order to better understand the epidemiological and evolutionary patterns of recent GII.P7_GII.6 viruses, we analysed a selection of GII.P7 noroviruses with GII.6, GII.7 and GII.14 genotypes identified during hospital-based surveillance in Italy. Also, in order to provide a spatio-temporal context to the epidemiological pattern observed locally, we extended the analysis to GII.6 and GII.P7 norovirus strains detected worldwide with sequences available in public databases.

2. Material and methods

2.1. Origin of samples

ISGEV conducts hospital-based surveillance on enteric viruses in Parma (Northern Italy), Bari (Southern Italy) and Palermo (Sicily) (Giammanco et al., 2013; Martella et al., 2013; De Grazia et al., 2013, 2018). The three hospitals serve a cumulate pediatric population (0–14 years of age) of 421,688 individuals (i.e. 5.1% of the total Italian pediatric population as of November 21, 2017). Between January 2014 and December 2016, ISGEV surveillance for norovirus analysed a total of 5853 stool samples of children hospitalized for AGE and identified 694 norovirus infections (11.9%). A total of 462 strains (66.6%) were multi-typed (350, 75.8%) in the ORF1 (polymerase type) and ORF2 (capsid type), whilst 112 (24.2%) were typed partially, in at least one target. Norovirus-positive samples were characterized by sequence analysis of region A (ORF1, RNA polymerase: 327 nt, at positions 4538–4865 relative to U07611 reference) and C (ORF2, capsid: 342 nt, at positions 5307–5649 relative to U07611 reference) using the Noronet automated genotyping tool (http://www.rivm.nl/mpf/typingtool/norovirus/). The GII.6 capsid type accounted for 5.3% (5/95) of norovirus infections in 2014, 10.0% (11/110) in 2015 and 3.8% (9/235) in 2016. The GII.P7 polymerase type accounted for 1.9% (2/101) of norovirus infections in 2014, 10.5% (9/86) in 2015 and 5.4% (10/185) in 2016. When polymerase and capsid typing data were merged, the prevalence of GII.P7_GII.6 viruses was 2.2% (2/90) in 2014, 8.8% (7/80) in 2015 and 3.9% (7/180) in 2016. The GII.6 capsid type was identified only in association with the GII.P7 polymerase type, whilst the GII.P7 polymerase gene was also found in combination with GII.7, GII.14 and GII.17 capsid types. In 2015, the GII.6 was the second most common capsid type after GII.4 (60.9%). The apparent increase of GII.6 prevalence in 2015 was statistically significant when compared to 2016

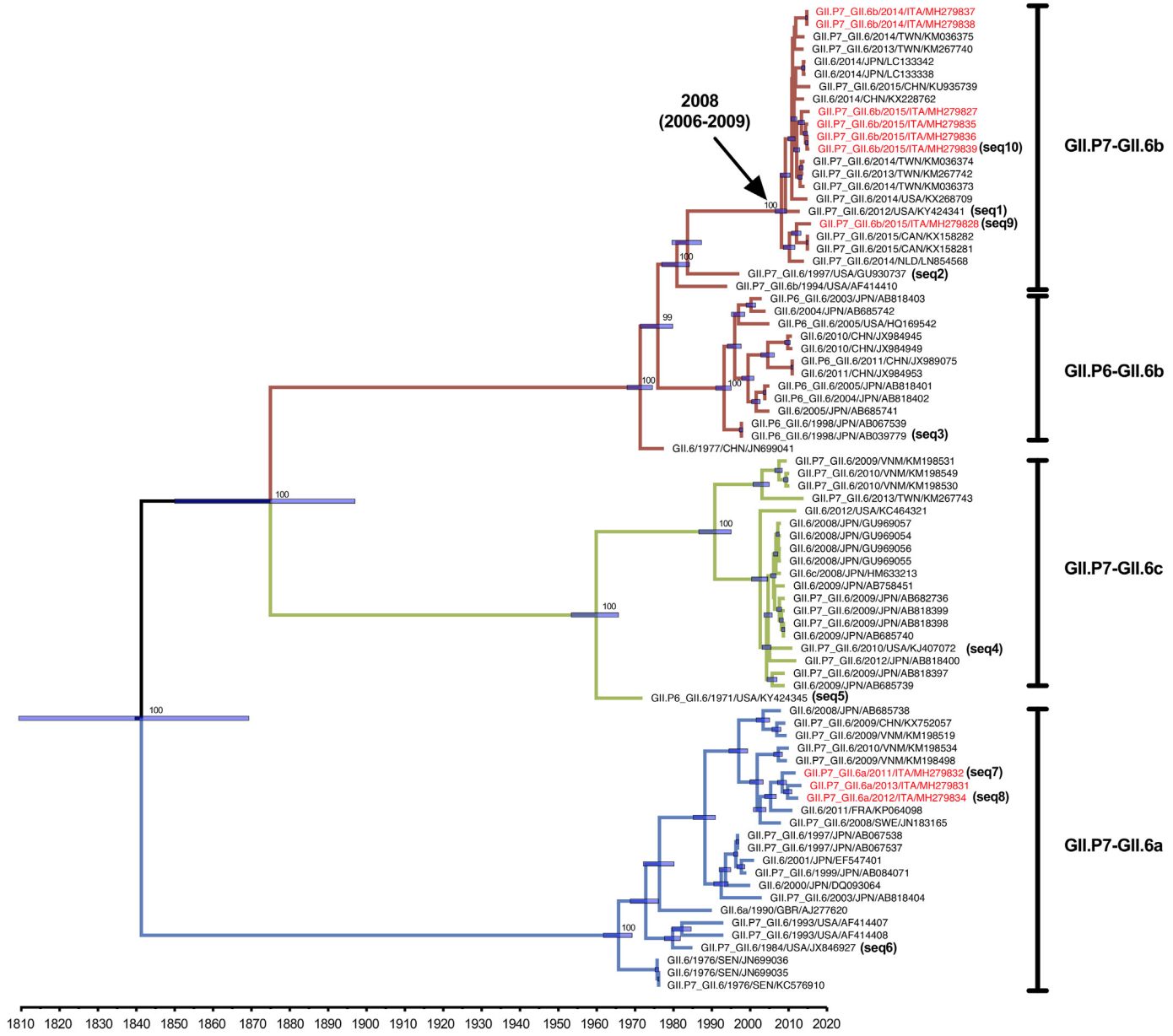

Fig. 1. Phylogenetic tree generated using the full-length (about 1600 nt) capsid gene (ORF2), of GII.6 noroviruses. The MCC tree was generated using 79 GII.6 sequences. The arrow indicates the most recent common ancestor of the sequences GII.P7_GII.6b circulating after 2012.The strains used as sequence type (listed in Table 2) are indicated in brackets. The strains sequenced in this study are indicated in red. The x axis indicates the time frame in year decades. Values on the major branches represent the posterior probability to support the nodes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

($p < 0.05$; χ^2 test), but not to 2014.

2.2. RNA extraction and amplification

Viral RNA was extracted from 140 µl of 10% *w/v* stool suspension using the QIAmp viral RNA kit (Qiagen, GmbH, Hilden, Germany). A 3′ RACE-PCR (rapid amplification of cDNA ends-PCR) protocol was used to generate the 3.2-kb amplicon encompassing the 3′ end of ORF1, the full-length ORF2 and ORF3, and the 3′ untranslated region (UTR) through the poly-A tail. Briefly, cDNA was synthesized by the SuperScript III First-Strand cDNA synthesis kit (Invitrogen Ltd., Paisley, United Kingdom) with primer VN3T20 (5′-GAGTGACCGCGGCCGCT20-3′) (Scotto–Lavino et al., 2006). PCR was then performed with TaKaRa La Taq polymerase (TaKaRa Bio Europe SAS, Saint-Germain en-Laye, France) with forward primer JV12Y (Vennema et al., 2002) and the reverse primer VN3T20.

2.3. Sequence and phylogenetic analyses

The amplicons were purified and cloned using the TOPOXL cloning kit (Invitrogen Ltd., Paisley, United Kingdom). Additional primers were designed to determine the complete 3.2-kb sequence by an overlapping strategy. Sequence editing and multiple codon based (translation) alignments were performed with Geneious software v.10.2.4 (Biomatters Ltd., Auckland, New Zealand). A 3.2-kb region of the genome at the 3′ end was determined for seven GII.P7_GII.6 strains collected in 2014–2015 and for three GII.P7_GII.6 strains detected in 2011–2013. Also, the same region was determined for three GII.P7_GII.7 and for a GII.P7_GII.14 strain (Table 1).

For sequence analysis, sets of full-length ORF2 sequences of GII.6,

Table 3
Comparison based on the nt and aa ORF2 sequence of GII.6 Italian viruses. The identity values are expressed as %. The highest identities values are shown in bold.

	GII.6a[a] AJ277620	GII.6a[b] KU870455	GII.6b[a] AF414410	GII.6b[b] KX268709	GII.6c[a] HM633213	Polymerase	Capsid
	nt/aa	nt/aa	nt/aa	nt/aa	nt/aa		
IT/2011/150/16-PA309	92.0/98.0	**98.5/99.1**	79.5/90.2	78.8/90.4	80.3/91.5	GII.P7	GII.6a
IT/2012/150/16-PA455	91.7/98.2	**98.1/99.5**	79.4/90.4	78.7/90.5	79.9/91.6	GII.P7	GII.6a
IT/2013/150/16-PA226	92.0/98.2	**98.4/99.5**	79.4/90.4	78.9/90.5	79.6/91.6	GII.P7	GII.6a
IT/2014/528/15-BA11	79.1/90.2	78.7/89.8	92.7/98.0	**98.3/99.3**	83.3/94.3	GII.P7	GII.6b
IT/2015/613-15-BA3	79.1/90.0	78.7/89.5	92.7/97.8	**98.4/99.1**	83.8/94.3	GII.P7	GII.6b
IT/2015/613/15-BA8	79.1/89.8	78.5/89.3	92.7/97.8	**98.5/99.1**	83.7/94.1	GII.P7	GII.6b
IT/2014/613/15-BA16	79.1/90.2	78.7/89.8	92.7/98.0	**98.3/99.3**	83.3/94.3	GII.P7	GII.6b
IT/2015/886/15-BA7	79.1/90.0	78.6/89.5	92.8/98.0	**98.5/99.3**	83.8/94.3	GII.P7	GII.6b
IT/2015/159/16-PR3020	79.1/90.2	78.5/89.6	92.9/98.4	**98.4/99.3**	83.6/94.5	GII.P7	GII.6b
IT/2015/159/16 PR4741	78.9/90.4	78.8/90.0	92.0/97.6	**96.9/98.7**	83.4/94.1	GII.P7	GII.6b

The highest identities values are shown in bold.
[a] Reference norovirus strains (Vinjé, 2015).
[b] Norovirus strains with higher % identity to the Italian strains retrieved from databases.

GII.14 and GII.7 viruses and of partial (about 800 nt in length) ORF1 sequences of GII.P7 viruses were retrieved from GenBank database. All the sequences obtained from GenBank were genotyped with the Noronet genotyping tool (http://www.rivm.nl/mpf/typingtool/norovirus) to rule out possible errors of annotation. The reference panel of ORF1 and ORF2 sequences defined by Noronet and Calicinet was used, as outlined elsewhere (Vinjé, 2015).

Time-measured phylogenetic analysis was performed using Bayesian Markov Chain Monte Carlo (MCMC) framework as implemented in BEAST v1.8.4 (Drummond et al., 2012). The SRD06 model was used for estimating the substitution process of the ORF1 and ORF2 sequences (Shapiro et al., 2006), and strict clock was applied to both of the datasets. The population size was assumed to be constant throughout their evolutionary history. The MCMC runs were performed until the convergence of all the parameters was confirmed by the effective sample size ($\geq$200 in all parameters) using Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/). The first 10% of the logs from the MCMC runs were removed as a burn-in, and the posterior trees were summarized as a Maximum Clade Credibility (MCC) tree using TreeAnnotator v1.8.3.

2.4. Recombination analysis

In order to clarify the genetic relationships among GII.P6_GII.7 noroviruses and to unveil possible intra-genotypic recombination, we selected representatives of the various GII.P6 and GII.P7 sub-clusters, as defined by ORF1 and ORF2 co-phylogenetic analysis. Conserved combinations of polymerase/capsid lineages were individuated and referred to as sequence (seq) types 1 to 6 and analysed along with a selection of Italian sequences (seq7 to seq10) (Table 2). The sequences were aligned and therefore analysed by Simplot 3.5.1 (Lole et al., 1999).

2.5. Sub-typing of GII.6 noroviruses in GenBank

A total of 368 sequences were retrieved from GenBank and analysed using the Classify Sequences software (Geneious v10.2.4) that classifies the query sequences by aligning against all sequences in a reference database. Database 1 included the prototype variant strains GB/1990/Seacroft/GII.6a (AJ277620), US/1994/Miami292/GII.6b (AF414410) and JP/2008/Shizuoka/GII6c (HM633213). Database 2 included 7 sequences representative of the GII.6 seq types, as defined in this study on the basis of co-phylogenetic analysis of ORF1 and ORF2, in order to have at least 2 strains for each GII.6 variants (Table 2). Various sensitivity options and a minimum overlap of 200 nt between the query and the database sequences were set up. A medium sensitivity (cut-off 80% identity) was used to classify the sequences into the three variants GII.6a, -6b and -6c. A high sensitivity (cut-off 90% identity) was used to classify the sequences into seq types 1 to 7.

3. Results

3.1. Sequence and phylogenetic analysis of ORF2 of GII.6 noroviruses

A total of 368 GII.6 (ORF2) sequences were retrieved from GenBank database. Eighty-one sequences covered the full-length ORF2 while 287 were partial sequences. For a subset of GII.6 capsid sequences (n = 130) the ORF1 sequence was also available. In this subset, all GII.6 capsid sequences were in combination with either GII.P7 (116/130, 89.2%) or GII.P6 (14/130, 10.8%) polymerase types. Twelve ORF2 sequences with incomplete information, duplicates or sequences obtained from the same patient/outbreak were discarded and a total of 69 full-length ORF2 sequences were used for phylogenetic analysis. For 47/69 (68.1%) of the ORF2 sequences, the ORF1 sequence was also available. The set of 69 ORF2 sequences was used to analyze our GII.6 strains listed in Table 1.

Upon sequence and phylogenetic analysis based on the whole ORF2 sequence ($\simeq$1600 nt), GII.6 viruses formed three large groups, namely GII.6a, GII.6b and GII.6c (Fig. 1). The GII.6a group only consisted of viruses with GII.P7 polymerase, including the three 2011–2013 Italian viruses. This group included viruses dating back to the mid-1970s, although the Italian 2011–2013 viruses segregated with more recent viruses (identified from 2008 onwards in Europe and Asia). The closest relative (98.1–98.4% nt and 99.1–99.5% aa identity) to this group of Italian viruses was the Chinese strain NHBGR59 (GenBank accession No. KU870455) (Table 3).

The GII.6b group could be separated into two subgroups, one associated with GII.P6 and the other one with GII.P7. The latter subgroup also included all the seven 2014–2015 Italian GII.P7_GII.6 viruses and it was formed exclusively by viruses detected from 2012 onwards in North America, Europe and Asia. The time of the most recent common ancestor of that group dated back to 2008 (95% highest posterior density interval: 2006–2009). The Italian viruses within this group displayed the highest identity (96.9–98.5% nt and 98.7–99.3% aa) to the GII.P7_GII.6b norovirus strain US/2014/Maryland (GenBank accession No. KX268709) (Table 3).

Finally, the GII.6c group included two distinct subgroups, one encompassing GII.P6 viruses (dating back to the 1970s) and the other encompassing GII.P7 strains detected after 2008. None of the Italian GII.6 viruses sequenced in this study were characterized as GII.6c.

3.2. Analysis of ORF2 of GII.7 and GII.14 noroviruses

Fourteen GII.P7_GII.7 and seventeen GII.P7_GII.14 norovirus ORF2 sequences were retrieved from GenBank database. Upon sequencing

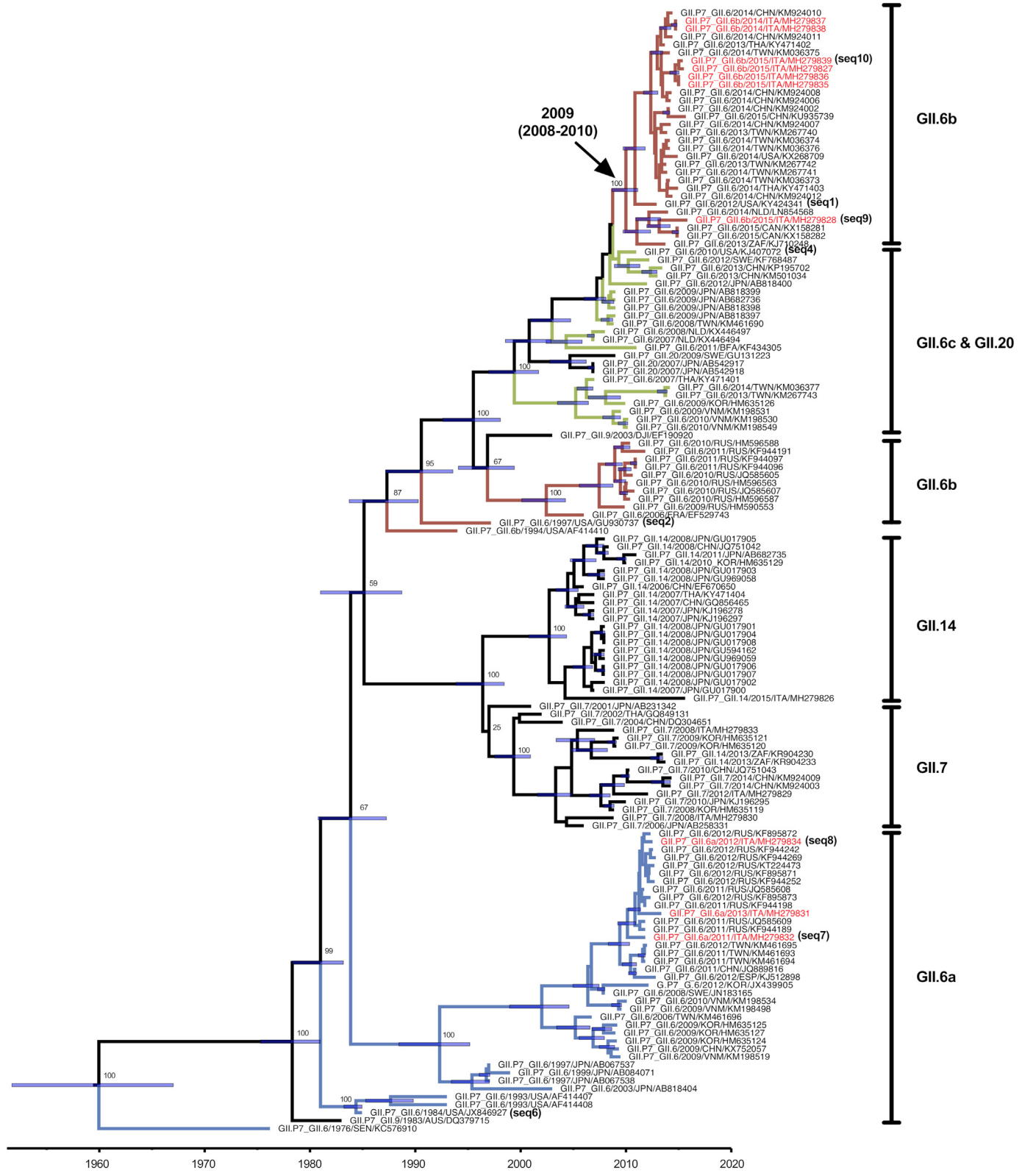

Fig. 2. Phylogenetic tree generated using the partial length (about 500 nt) RdRp region of ORF1, of GII.P7 noroviruses. The MCC tree was generated using 141 GII.P7 sequences. The arrow indicates the most recent common ancestor of the sequences GII.P7_GII.6b circulating after 2012.The strains used as sequence type (listed in Table 2) are indicated in brackets. The strains sequenced in this study are indicated in red. The x axis indicates the time frame in year decades. Values on the major branches represent the posterior probability to support the nodes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

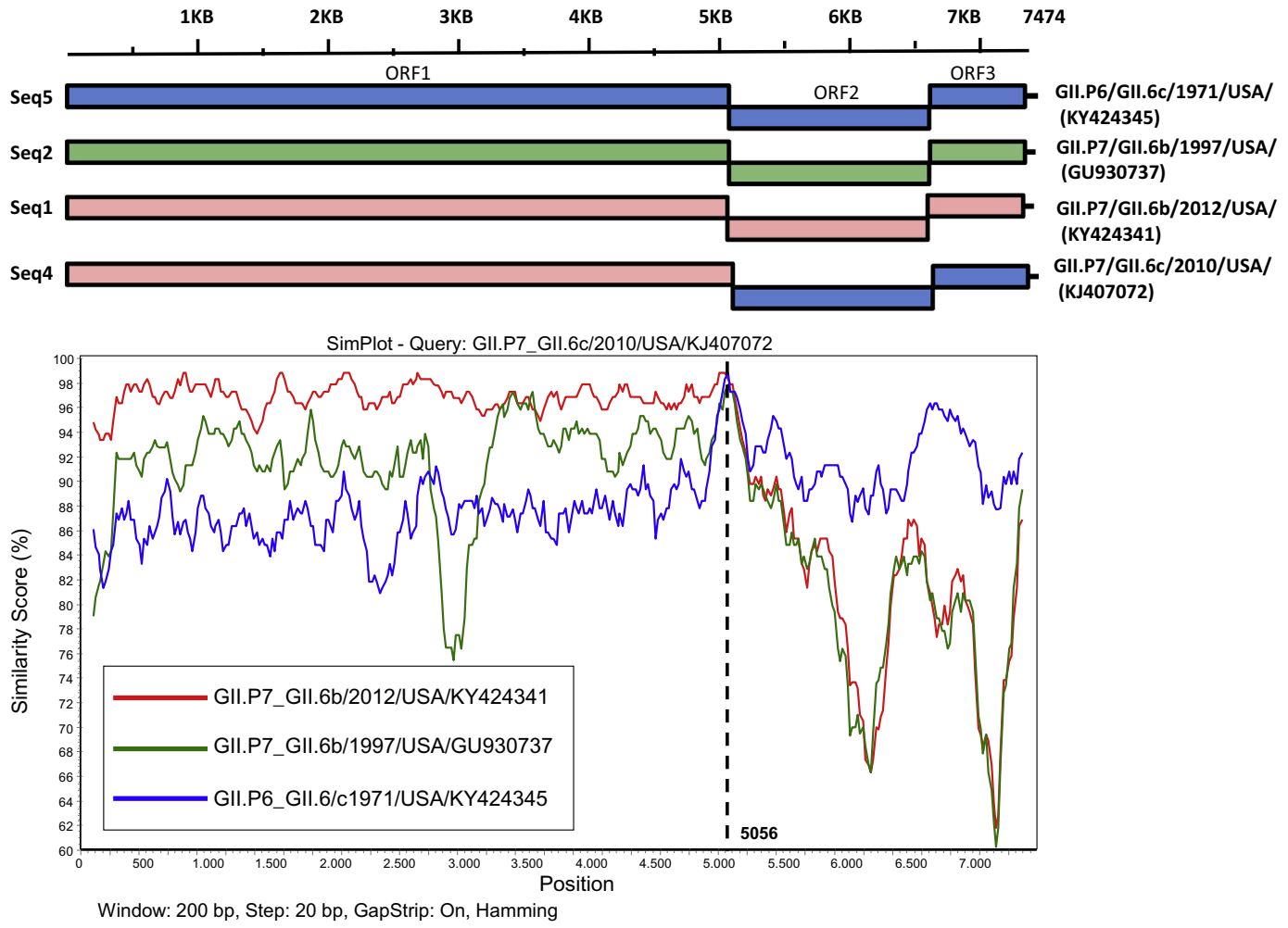

Fig. 3. Nucleotide identity plot of the genome of a GII.P7_GII.6c norovirus variant (seq4), from the 5′ UTR to the 3′ UTR. Seq4 genome was compared with the genome of recent (seq1) and old (seq2) GII.P7_GII.6b strains and of a GII.P6_GII.6c strain (seq5). The sequences were analysed with Simplot (Lole et al., 1999) using a window size of 200 and step size of 20, with gap strip off and Hamming correction on. The dotted line indicates the crossover site at the ORF1-ORF2 junction region. The colors used in the schematic representation of the norovirus genomes indicate their genetic relationships. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

analysis, the GII.P7_GII.7 Italian strains displayed the highest identity (95.6–98.8% nt and 98.3–98.7% aa) to the GII.P7_GII.7 strain NL/2013/Rotterdam/E1300273_p12_d0 (GenBank accession No. MF140645) whilst the GII.14 virus displayed the highest identity (96.1% nt and 98.9% aa) to the GII.P7_GII.14 strain JP/2008/Maizuru/8594 (GenBank accession No. GU017907).

3.3. Analysis of ORF1 of GII.P7 noroviruses

We retrieved from GenBank database a total of 160 GII.P7 (ORF1) sequences associated to the following capsid genotypes: GII.6 (116/160, 72.5%), GII.7 (16/160, 10%), GII.9 (3/160, 1.9%), GII.14 (22/160, 13.8%) and GII.20 (3/160, 1.9%). A subset of 127 representative sequences was analysed along with the polymerase sequences of the 14 Italian GII.P7 viruses. Phylogenetic analysis of GII.P7 viruses, based on a 498-nt long fragment of the ORF1 (Fig. 2), revealed a pattern of co-segregation for GII.6a noroviruses, including the 2011–2013 Italian strains. A large group of GII.P7 polymerase sequences included exclusively noroviruses with either GII.7 or GII.14 capsid sequences. This group also included the Italian GII.P7_GII.7 and GII.P7_GII.14 viruses. Finally, a large group of GII.P7 polymerase sequences included GII.6b (42/127, 33.1%) and GII.6c (20/117, 17.1%) viruses, along with GII.20 (3/117, 2.6%) recombinant strains. Within this large group, GII.6b viruses segregated within two distinct GII.P7 polymerase sub-groups, with the Italian GII.6b viruses being included in a well-defined group formed exclusively by viruses detected after 2012.

3.4. Recombination analysis

Simplot analyses of the ten selected strains, including noroviruses of the various GII.6 sub-clusters in association with GII.P6 or GII.P7 (seq1 to seq6) and the four Italian GII.6 viruses (seq7 to seq10) showed in Table 2, revealed a clear pattern of recombination between GII.P7_GII.6b variant BethesdaD1 2012-like viruses (seq1) and GII.P7_GII.6c viruses (seq4), which shared a highly related polymerase gene (Fig. 3).

3.5. Sub-typing of GII.6 noroviruses in GenBank

A set of 368 GII.6 sequences retrieved from GenBank was analysed using two different reference sequence sets for data analysis. The initial analysis was carried out using the prototype variant strains GB/1990/Seacroft/GII.6a (AJ277620), US/1994/Miami292/GII.6b (AF414410) and JP/2008/Shizuoka/GII6c (HM633213). In this analysis, 138 (37.5%) sequences were classified as GII.6a, 127 (34.5%) as GII.6b and 103 (28.0%) as GII.6c (Fig. 4a). Further analysis was done using a panel of seven ORF2 seq types that were defined on the basis of the co-

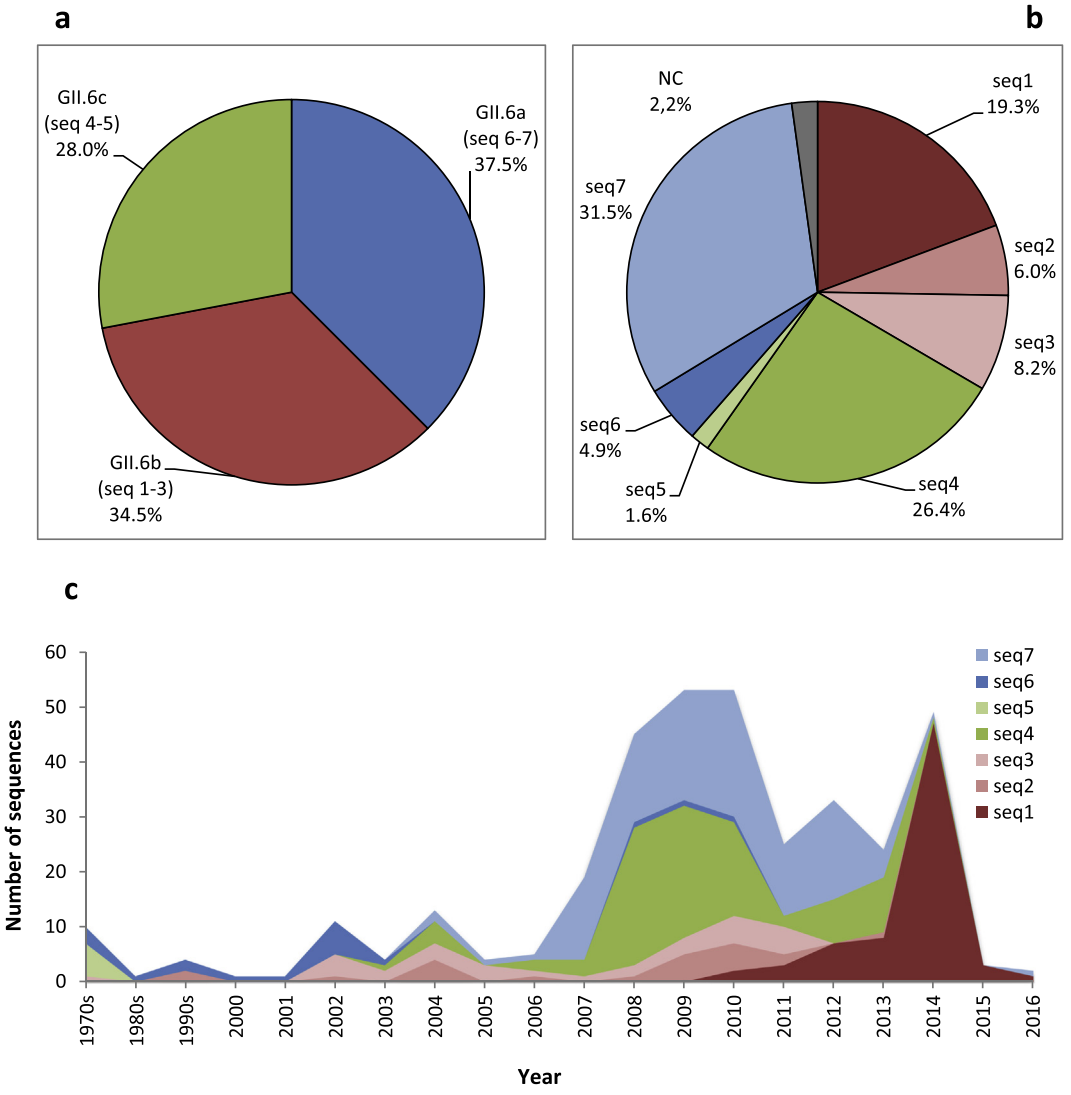

Fig. 4. Classification of GII.6 norovirus sequences using pairwise global alignment with Sequence Classifier software. Panel a: distribution of GII.6 sequences using the prototype variant strains GB/1990/Seacroft GII.6a (AJ277620), US/1994/Miami292/GII.6b (AF414410) and JP/2008/Shizuoka/GII6c (HM633213) as reference data set. Panel b: distribution of GII.6 sequences using sequence types (seq1 to seq7) (listed in Table 2) as reference data set. Abbreviations: NC, non-classified. Panel c: temporal dispersion of GII.6 seq types. Due to the limited number of sequences released for the years 1971–1979, 1980–1989 and 1990–1999, the data before 2000 were grouped by decades. The Y axis indicates the number of sequences. The X axis indicates the year of collection.

phylogenetic analysis of ORF1 and ORF2 (Table 2). At least two strains for each GII.6 variant, representative of recent and older viruses, and/or of different capsid/polymerase combinations were also included. In this analysis, 71 sequences were typed as GII.P7_GII.6b seq1 (KY424341), 22 as GII.P7_GII.6b seq2 (GU930737) and 30 as GII.P6_-GII.6b seq3 (AB039778), 116 as GII.6a seq7 (MH279832) and 18 as GII.6a seq6 (JX846927). Of the 103 GII.6c strains, 97 were typed as GII.P7_GII.6c seq4 (KJ407072) and 6 as GII.P6_GII.6c seq5 (KY424345) (Fig. 4b).

A temporal pattern of distribution was observed for the various seq types when considering the year of collection (Fig. 4c). In particular, GII.P7_GII.6c appeared after 2002, GII.P7_GII.6a after 2003 and GII.P7_GII.6b appeared after 2010. The studies reporting the circulation of GII.6b BethesdaD1-like viruses, the year of detection and the geographic origin are listed in detail in Table 4. Of note is the limited availability of data prior to 2000s.

4. Discussion

Noroviruses with GII.6 capsid type were first reported in a nosocomial outbreak in Henryton, Maryland, US, in December 1971. The outbreak caused by the GII.P6_GII.6c strain US/1971/HenrytonH1 affected 64/382 (17%) patients (Karangwa et al., 2017) and occurred 11 months apart from a GII.2 norovirus outbreak that occurred in the same hospital, suggesting limited cross-protective immunity between these two norovirus genotypes (Parra and Green, 2014; Parra et al., 2017). Upon sequence and phylogenetic analysis, GII.6 noroviruses have been distinguished into at least three sub-types, namely GII.6a, GII.6b and GII.6c (Chan-It et al., 2012). Strains within each of these groups tend to be conserved as they display < 5% aa variation (Parra et al., 2017). Three reference GII.6 strains have been proposed (Vinjé, 2015), namely the GII.6a strain GB/1990/GII.6/Seacroft (GenBank accession No. AJ277620), the GII.6b strain US/1994/GII.6/Miami292 (GenBank accession No. AF414410) and the GII.6c strain JP/2008/GII.6/Shizuoka (GenBank accession No. HM633213). Phenotypic differences in terms of antigenicity (Lindesmith et al., 2008), ability to bind human HBGAs co-receptors (Tan and Jiang, 2014) and/or virus fitness (van Beek et al., 2013) have been observed between GII.4 variants. Likewise, changes in the carbohydrate binding properties of GII.3 norovirus VLPs from each of the years 1975, 1976, 1988, 1990, 1991,

Table 4
List of studies reporting the circulation of GII.6b noroviruses BethesdaD1-like (seq1). The country, number of available GII.6b sequences, the prevalence of GII.6 noroviruses, the study period and reference are indicated. NA: information not available.

Country	Nr	Year of collection	GII.6 prevalence	Study years	Reference
Israel	2	2010	3/26 (11.5%)	2010	Muhsen et al., 2013
Lebanon	2	2011	7/76 (9.2%)	2011–13	Melhem et al., 2016
Lebanon	2	2012	–	–	Melhem et al., 2016
Russia	1	2011	20/892 (2.2%)	2003–12	Zhirakovskaia et al., 2015
Russia	7	2014	NA	NA	Unpublished
Thailand	1	2012	4/104 (3.9%)	2009–14	Phumpholsup et al., 2015
Thailand	2	2013	–	–	Phumpholsup et al., 2015
Thailand	1	2013	17/1938 (0.9%)	2005–15	Supadej et al., 2017
Thailand	1	2014	–	–	Supadej et al., 2017
Brazil	1	2013	2/57 (3.5%)	2013–14	Fumian et al., 2016
Brazil	1	2016	NA	NA	Unpublished
New Zealand	1	2013	14/344 (4.1%)	2013–14	Lim et al., 2016
New Zealand	7	2014	–	–	Lim et al., 2016
Belgium	1	2014	6/533 (1.1%)	2004–14	Wollants et al., 2015
Brazil	2	2014	3/14 (21.4%)	2014–15	Dábilla et al., 2017
China	2	2014	2/25 (8%)	2014	Wang et al., 2015
China	1	2015	NA	NA	Huo et al., 2017
China	9	2014	NA	NA	Unpublished
Japan	8	2014	8/101 (7.9%)	2013–16	Kazama et al., 2017
Japan	3	2014	NA	NA	Unpublished
USA	4	2012	NA	1995–2015	Parra et al., 2017
USA	1	2014	98/893 (11%)[a]	2014–15	Yang et al., 2016
Taiwan	2	2013	NA	NA	Unpublished
Taiwan	5	2014	NA	NA	Unpublished
South Africa	1	2013	6/339 (1.8%)	2009–13	Mans et al., 2014
The Netherlands	1	2014	1/10 (10%)	NA	Bavelaar et al., 2015
Canada	2	2015	4/8 (50%)	2013–15	Nasheri et al., 2017

[a] Outbreaks.

1996 and 2001 have been demonstrated (Boon et al., 2011). Whether the observed genetic heterogeneity is associated with antigenic or other biological differences among the various GII.6 strains has not been investigated. Interestingly, GII.P7_GII.6b noroviruses (BethesdaD1 2012-like) seem to have the ability to bind to salivary HBGAs derived from a wide spectrum of blood type A, B, AB and O individuals (Zheng et al., 2017).

In this study, we analysed GII.6 norovirus strains circulating in Italy between 2011 and 2016. The rationale for the study was the increased circulation of GII.6 noroviruses monitored by ISGEV in 2015, when these viruses accounted for about 10% of the norovirus-confirmed hospitalizations for AGE. Although the observed year-to-year variations were barely statistically significant, and might merely reflect spatial-temporal fluctuations, large epidemiological investigations by surveillance networks and systematic review of norovirus literature indicate that GII.6 noroviruses are among the most common norovirus strains after GII.4 (Hoa Tran et al., 2013; Bruggink et al., 2017; Cannon et al., 2017; van Beek et al., 2018).

By analysing a selection of GII.6 noroviruses detected in Italy between 2011 and 2016, the oldest (2011 − 2013) Italian GII.6 viruses, detected only sporadically, were all classified as GII.6a variant, whilst recent GII.6 viruses (2014–2016), detected more frequently in the population, were classified as GII.6b. A comprehensive analysis of GII.P7 and GII.6 noroviruses revealed that the GII.P7 polymerase was found in combination with all GII.6 capsid variants. In turn, the GII.P6 strains were detected in combination with GII.6b and GII.6c, but not with GII.6a capsid sequences. This observation suggests multiple recombination events between GII.P7 and GII.6 viruses, rather than a linear evolution from a common recombinant GII.P7_GII.6 ancestor (Tohma et al., 2018).

Within this recent group of GII.6b viruses, the oldest GII.P7_GII.6b norovirus with a complete capsid sequence available in GenBank was a strain detected in US in December 2012 (strain US/2012/BethesdaD1, accession no. KY424341), whilst two older GII.6b strains, dating back to 1994 and 1997, formed distinct side branches with respect to the new GII.6b viruses. Interestingly, both co-phylogenetic and recombination analysis revealed that strain US/2012/BethesdaD1 (seq1 type) showed a GII.P7 polymerase gene highly genetically related to that of GII.P7_GII.6c viruses (seq4 type), suggesting a recombinant origin for the strain (Fig. 3).

Norovirus classification requires continual updates as novel candidate genotypes are continuously being discovered (van Beek et al., 2018; Chhabra et al., 2018). A hot spot for norovirus recombination is the highly conserved ORF1/ORF2 junction region (Bull et al., 2005), where recombination occurs preferentially, but not exclusively. Considering only the GII human noroviruses, at least 19 capsid types and 24 polymerase types have been described (Vinjé, 2015), with a total of 456 inter-genogroup possible combinations. Accordingly, the apparent co-segregation of the GII.6 capsid with the GII.P7 polymerase seems of difficult explanation. Co-segregation patterns or genetic linkages able to select preferentially some genome segments have been hypothesized for rotaviruses (Iturriza-Gòmara et al., 2003). Alternatively, this linkage could be due to the high genetic relatedness among strains with this polymerase and capsid combination, as recombination occurs more frequently between highly genetically related viruses than between distantly related norovirus strains (Bull et al., 2012). For instance, intra-genotypic recombination among GII.4 viruses is a driving force creating GII.4 variants (Eden et al., 2013). Since the emergence of the GII.4 variant Farmington Hills 2002, all subsequently identified GII.4 variants have acted as the parental strain or as a product of a recombination event. This genetic mixing was likely favored by the prolonged circulation of some GII.4 variants. The latest variant Sydney 2012, GII.Pe_GII.4, was hypothesized to have derived from previous variants (Hunter 2004, Yerseke 2006a, Cairo 2007, Osaka 2007, Den Haag 2006b, Apeldoorn 2008 and New Orleans 2009) throughout multiple recombination events (Eden et al., 2013). Moreover, shortly after its emergence, the Sydney 2012 variant generated recombinants with a GII.P4 polymerase derived from the New Orleans 2009 variant (Martella et al., 2013), and more recently, acquired the GII.P16 polymerase gene, likely from a GII.2 norovirus (Cannon et al., 2017).

In this study, the relative and temporal distribution of the three GII.6 variants in the database was investigated, using all GII.6

sequences available. Upon analysis of the sequence data, we found out that the three variants were almost equally represented with GII.6a, GII.6b and GII.6c accounting for 37.5%, 34.5% and 28.0%, respectively, of the recognised sequences (Fig. 4a). When trying to assess the relative distribution of the seq types of the three GII.6 variants, we noted that GII.6b seq1 strains (Bethesda 2012-like), the GII.6c seq4 strain and the recent GII.6a viruses (seq7) were over-represented (Fig. 4b). Upon analysis of a 368-sequence set, we observed that GII.6b seq1 norovirus strains (BethesdaD1 2012-like) were circulating as early as 2010 whilst GII.6b seq2 noroviruses were no longer identified after 2013, suggesting a temporal pattern of replacement (Fig. 4c). This kind of analyses and hypotheses inevitably rely on the availability of adequate amount of sequences in the database; therefore, our analysis could be affected by strong geographical and temporal sampling biases, which hamper an exact reconstruction of the evolution pathways followed by GII.6 noroviruses. Interestingly, the BethesdaD1 2012-like GII.6b viruses were reported from countries of virtually all continents (Europe, Africa, Asia, Australia, Northern and Southern America), supporting the notion that once a norovirus emerge, could rapidly spread worldwide (Table 4).

Overall, our findings indicate that the GII.P7_GII.6b strains that started circulating since 2014 in Italy were derived from the introduction of a recent norovirus strain, the GII.6b strain BethesdaD1 2012, that emerged globally after 2008. Continual surveillance of noroviruses is needed to monitor changes in norovirus incidence and genetic diversification, as these changes could affect, to a significant extent, the diagnostic assays and the prophylaxis strategies, chiefly when devising novel vaccines against non-GII.4 noroviruses.

Declaration of Competing Interest

The authors declare no conflict of interest.

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